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Sensitive Radioimmune Assay for Measuring Aleutian Disease Virus Antigen and Antibody

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A solid-phase, one-step radioimmune assay was developed which could detect as little as 0.02 μl of a standard Aleutian disease virus antigen preparation, approximately 3.2 ng of viral protein. Virus antigen was measured in different mink organs and cell types during experimental intraperitoneal infection. The gut and kidney were the first organs in which virus antigen could be detected (day 3 to 6 after infection). On day 6 or later virus antigen was found in spleen, liver, kidney, lymph nodes, peritoneal exudate, and bone marrow cells. With inhibition of antigen binding, a radioimmune assay was developed for antibody detection. Viral antibodies could be detected as early as 3 days after infection. Antibody titers from $1/10^5$ to more than $1/10^6$ were found in plasmacytotic mink. When the sensitivity of the antibody radioimmune assay was compared with that of other known methods for anti-Aleutian disease virus quantitation, the radioimmune assay was considerably more sensitive, detecting as little as 5 ng of antibody.

Aleutian disease (AD) of mink is a progressive, usually fatal, immune complex disease induced by the Aleutian disease virus (ADV), a nondefective parvovirus (for review, see references 13 and 15). AD is of interest from practical as well as theoretical standpoints. AD causes substantial economic losses to the fur industry, and herds can be freed from AD if seropositive animals are removed. Consequently, eradication programs in which mink reacting to ADV antigen in countercurrent electrophoresis are culled have been developed and are highly successful (6). Nevertheless, false-positive reactions from AD-free farms occur, and development of a more sensitive and reliable assay for anti-ADV antibody would be desirable.

The pathogenetic features of AD are also of interest. The highly virulent Utah I strain of ADV has maximal virus titer on day 10 after experimental infection (14). The antibody titer against ADV and serum γ-globulin in general increase steadily after infection, and the mink become hypergammaglobulinemic. Immune complexes develop, and the mink suffer from glomerulonephritis and arteritis (14). The study of AD, however, has been limited due to difficulties in assaying virus from infected animals. Titrations of virus in animals are cumbersome, lengthy, and expensive, and the assay of infectious virus from tissues in culture is generally unsatisfactory. There has therefore been a demand for a method for detection and quantitation of ADV antigen. The known methods for ADV detection such as countercurrent assay or rocket electrophoresis are not very sensitive. Fluorescence microscopy has been used in detection of ADV antigen (14), but autofluorescence of phagocytic cells is known to give high background reactivity in tissues, frequently making the results difficult to interpret. Thus, not very much is known about the concentration of ADV in different organs and cells during infection. Furthermore, it has been impossible to test any theories concerning the disturbance of the immune apparatus, which leads to the progressive immune complex disease. The only findings with regard to ADV detection in vivo have been the detection of ADV antigen in the cytoplasm of Kuppfer cells (14) and other phagocytic cells. There is no direct proof that these cells are the target cells for ADV replication. The other major report on ADV quantitation in vivo (10) found mesenteric lymph nodes, spleen and kidneys to be probable sources of virus.

In the present study, we have developed a radioimmune assay (RIA) method for quantitation of ADV antigen in organ and cell homogenates from infected mink. Some organs, especially lymphoid organs, were found to contain ADV antigen.

We have also developed a fast and sensitive RIA for antibody quantitation, which detects as little as 5 ng of antibody to ADV.

MATERIALS AND METHODS

Animals. Sapphire mink (genotype *a/a*) and black mink (genotype *A/A*) were kept in isolated cages and fed a standard mink diet. For experimental ADV infections, only ADV-negative sapphire mink were used. Black mink were naturally infected with the DK strain of ADV. All other mink were experimentally infected.

ADV strains. The Utah I, Pullman, DK, and ADV-G strains of ADV used in this study have been described before (1, 3, 4). Intraperitoneal inoculation doses were 10^7 50% infective doses for Utah I, $10^{6.5}$ 50% infective doses for Pullman, 0.1 ml of DK countercurrent electrophoresis antigen (1), and various doses of ADV-G (4).

ADV standard antigen preparation. Five sapphire mink were infected with 10^7 50% infective doses of Utah I ADV and killed on day 10. A 20% (wt/vol) homogenate of liver, spleen, and lymph nodes in phosphate-buffered saline (PBS; 0.02 M phosphate, 0.13 M NaCl, 0.1% NaN_3 [pH 7.3]) containing 10 U of Trasylol (Mobay Chemical Corp., FBA Pharmaceuticals, New York, N.Y.) per ml was prepared by homogenizing for 3 min in a Waring blender. The homogenate was frozen and thawed four times and sonicated for 0.5 min. One-half volume of Freon 113 was blended with the homogenate for 3 min, followed by centrifugation at 2,500 × *g* for 0.5 h. The supernatant fluid was then centrifuged for 1 h at 234,000 × *g*, and the pellet was suspended in 1/5 volume of PBS-Trasylol buffer. Four consecutive Freon treatments were then done. Finally, the antigens were digested with DNase (250 μg/ml; P/L Biochemicals, Milwaukee, Wis.) in the presence of 0.01 M $MgCl_2$ for 60 min at 37°C.

ADV antigen quantitation in organs and cell types. Small quantities of organs and cell suspensions were homogenized in Dounce homogenizers. In certain experiments lymph node cells were liberated from the tissue by passing the tissue through a stainless steel screen. Peritoneal exudate cells were obtained by intraperitoneal infusion of 50 ml of PBS0.002 M EDTA buffer (without azide) in ether-anesthetized mink. After massage of the peritoneum, as much as possible of the infused solution was collected. Bone marrow cells were collected by flushing the femurs with PBS. Lymphocytes, peritoneal exudate, and bone marrow cells were washed three times and suspended in ~1 ml of PBS-Trasylol buffer. From this step on the cells were treated as the organs according by the procedure described above (ADV standard antigen preparation), except that only a limited number of Freon treatments was performed.

Sera. Blood was taken by cardiac puncture, and serum was separated by centrifugation for 5 min at 2,500 × *g* after overnight clotting. Serum pools were prepared from 5 to 20 plasmacytotic mink after infection with each of the mentioned ADV strains. A normal serum pool was prepared from 20 mink.

Immunoglobulin G (IgG) purification. Anti-Pullman serum was passed through staphylococcal protein A-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) and extensively washed with PBS until the protein absorbance at 280 nm was less than 0.01. The adsorbed protein was eluted with 0.5 M acetic acid and dialyzed immediately against PBS. The final preparation was analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol (4) and found to be more than 95% pure heavy and light chains, as judged by staining with Coomassie blue.

Protein determination and serum γ-globulin quantitation. Total serum protein was quantitated by refractometry. Sera were electrophoresed on cellulose acetate membranes (Microzone system; Beckman Instruments, Fullerton, Calif.), and the γ-globulin fraction was calculated on the basis of total protein. The protein concentration of the purified IgG preparation was calculated on the basis of 1.500 as the extinction coefficient for 1 mg of mink γ-globulin per ml.

[^{125}I]IgG preparation. The IgG preparation from the antiserum against Pullman ADV was used for iodination. Iodination was performed by the iodogen method (11).

Immunofluorescence assay. Indirect cytoplasmic immunofluorescence was done on monolayers of CRFK cells (8). Microscope slides (25 by 75 mm) were placed in large Leighton tubes and seeded with 1.5 × 10^5 CRFK cells in 5 ml of complete medium (4). The next day, the medium was removed and the subconfluent cell sheets were infected with 1 ml of medium containing a dilution of ADV-G that induced virus specific fluorescence in about 10% of the cells. Two hours later, 4 ml of complete medium was added, and the slides were incubated at 31.8°C for 3 days. Slides were removed, washed in PBS, fixed in acetone for 10 min, air dried, and stored at −20°C until use. Slides were divided into replicate compartments with pliobond (Goodyear Tire and Rubber Co., Akron, Ohio). Assays were performed by incubating compartments of the cell sheet with 10 μl of serum dilutions at 37°C for 30 min. Slides were washed 10 min in PBS and then overlaid with 10 to 20 μl of fluorescein isothiocyanate-conjugated rabbit antibodies against mink IgG (prepared as described previously [7]). After 30 min of incubation at 37°C, slides were again washed, dried, and examined in a Leitz Orthoplan microscope. The reaction of fluorescein conjugates of immunoglobulin prepared from sera of AD-affected mink was used as a positive control (4).

Countercurrent electrophoresis. Direct and indirect countercurrent electrophoresis were performed as previously described (2).

Antigen RIA (ag-RIA). The basic principle of the RIA is the formation of immune complexes consisting of solid-phase antibody-ADV antigen-radiolabeled antibody in a one-step reaction in which all reagents are mixed. If antigen is present, it will cross-link the isotope to the solid phase. Four different buffers were used all containing 0.1% azide. Adsorption buffer was PBS. Saturation buffer was PBS with 1% bovine serum albumin (bovine albumin powder; Metrix, Chicago, Ill.). Reaction buffers were PBS–0.1% Tween 20–0.35% gelatin or PBS–0.1% Tween 20–1% normal mink serum. Washing buffer was PBS–0.1% Tween 20.

The antigen-antibody reactions were carried out in large 96-well (2-ml wells) flat-bottom nonsterile trays

(76-302-05; Linbro Division, Flow Laboratories, Hamden, Conn.). The solid-phase RIA was performed as follows by using 1/4-in. (ca. 0.63-cm) polystyrene pearls from Precision Ball Co., Chicago, Ill.

Fifty milliliters of plastic pearls (approximately 200) plus 50 ml of PBS were deaerated for 1 min or longer before 25 μl of standard antiserum (anti-Pullman) was added. Adsorption times were from overnight to 1 week at 4°C. Before use the antiserum-treated pearls were incubated with saturation buffer for at least 0.5 h at 37°C, after which they were washed three times in washing buffer. One hundred nanograms of [^{125}I]IgG (approximately 100,000 cpm) and one antiserum-coated, protein-saturated, and washed pearl were added to each well in a volume of 400 μl of reaction buffer. Standard antigen or unknown samples were then added, and the total volume was brought up to 500 μl with reaction buffer.

Incubations were carried out at 37°C in a humidity chamber with orbital shaking (pearls in constant circular movement). Incubation time was normally 4 h. At the end of the incubation time, three washings (10 min each) were done at room temperature, after which the pearls were transferred to counting tubes and counted in a Beckman γ-counter.

ab-RIA. For the antibody RIA (ab-RIA), the buffers were the same as for the ag-RIA, except that only PBS–0.1% Tween 20–1% normal mink serum was used as the antigen-antibody incubation buffer (reaction buffer). The adsorption of antiserum to the plastic pearls and saturation process were the same as for the ag-RIA.

The procedure was as follows. One hundred nanograms of [^{125}I]IgG and one antiserum-coated pearl were added to each well in 400 μl of reaction buffer. Serum samples to be analyzed for ADV antibody were used, and the volume was brought up to 495 μl with reaction buffer. After a brief mixing, 5 μl of standard ADV antigen was added with a Hamilton syringe with dispensor. Each experiment always included reaction mixtures without antibody to obtain zero inhibition values. The zero inhibition binding values were identical to binding values of 5 μl of standard antigen in the ag-RIA. Incubation time was normally 2 h at 37°C. The washing and counting procedure was the same as for the ag-RIA.

To express the inhibitory effect of a given antiserum, the titer was estimated as the dilution of serum that produced 50% inhibition of binding.

Optimization of RIA conditions. Before the above final RIA conditions were achieved, studies were carried out to investigate and optimize several of the parameters included in the assay. These parameters were (i) the adsorption of protein (antibody) to and the elution of bound protein (antibody) from the pearls' plastic surface during incubation and (ii) RIA buffer composition, incubation time, and temperature. The following findings were obtained (data not shown).

(i) Protein adsorption to and elution from solid phase. A 1:2,000 dilution of standard antiserum used for plastic coating was shown to give the highest binding values. The amount of protein bound to the plastic surface after 3 days of incubation was determined by isotope quantitation to be 1.36 μg of protein per plastic pearl.

The protein saturation process where the plastic pearls were incubated with a 1% bovine serum albumin–PBS solution (0.5 h at 37°C) resulted in a loss of 13% of the protein. The 4-h incubation step (also at 37°C) resulted in loss of 62% of the protein originally bound. Thus, during saturation and incubation 75% of the protein was lost totally, leaving 340 ng of protein per plastic pearl (or 131 ng of γ-globulin because 38% of the total serum protein was γ-globulin).

(ii) RIA buffer composition, incubation time, and temperature. A 0.35% concentration of gelatin was found to give maximal antigen binding values. For antibody detection (see below), it was advantageous to use 1% normal mink serum instead of gelatin. No significant difference in antigen detection standard curves was found when the gelatin-containing buffer was compared with the normal mink serum-containing buffer. The nonionic detergent Tween 20 was also added to the buffer. A 0.1% Tween concentration was found to give the highest binding values. Incubations of 4 h or longer were found to give maximal binding values. ag-RIA standard assays performed at room temperatures required longer incubation times than those at 37°C to obtain maximal binding values, and therefore 37°C was used as the standard incubation temperature. For the ab-RIA, incubations of 2 h at 37°C were found to give optimal antibody titers, although longer incubation times could be used just as well.

RESULTS

Determination of ADV antigen by RIA. When a mixture of solid-phase antiserum to ADV (antiserum from Pullman ADV-infected mink coated onto plastic pearls) and 100 ng of [^{125}I]IgG (IgG purified from the same antiserum by staphylococcal protein A-Sepharose purification and subsequently radiolabeled) was mixed with increasing quantities of a standard ADV antigen preparation, increasing binding values of the isotope to the solid phase were observed (Fig. 1). The RIA could easily detect 0.02 μl of standard ADV antigen.

At the right end of the antigen standard curve a plateau was observed at 15,837 cpm. When antigen concentrations larger than those depicted in Fig. 1 were used, a decline in the binding values was observed probably because larger antigen concentrations prevented cross-linking of ^{125}I-labeled antibody to solid-phase antibody. Since 103,000 cpm of [^{125}I]IgG reagent was added to each reaction mixture, this plateau represented 15.4% of the total counts added. Thus, 15.4% of the IgG preparation used for radiolabeling bound to the ADV antigen; this suggested that 15.4% of IgG was virus-specific antibody.

Determination of antibody to ADV by inhibition of binding RIA. For detection of antibody to ADV, we used a modified ag-RIA. Antibody to ADV inhibited the ag-RIA, competing with solid-phase antibody and radiolabeled antibody for antigen. Inhibition curves for different concentrations of antisera are shown in Fig. 2. Normal

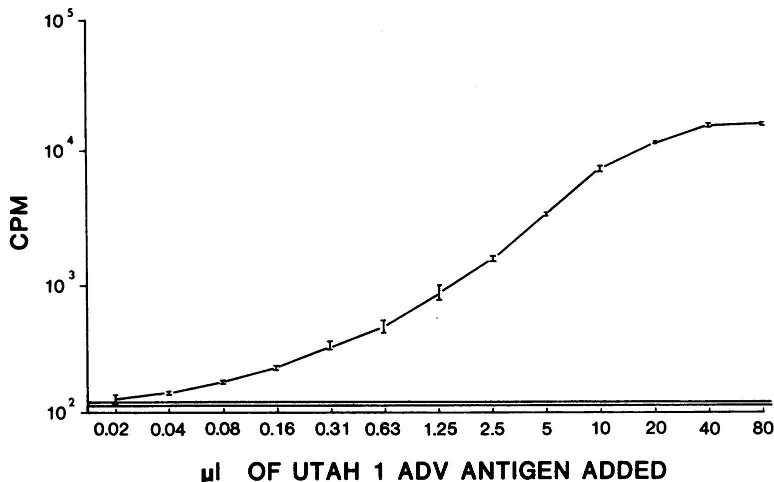

FIG. 1. Detection of different quantities of Utah I antigen by the RIA. Triplicate determinations were performed. The brackets indicate standard deviations. The standard deviation of multiple samples without any antigen (background value) is indicated by the two parallel lines. The estimated protein concentration of ADV antigen in the antigen preparation used was 161 µg/ml.

mink serum in concentrations from 20% (0.5×10^1, Fig. 2) to a little less than 1% (10^2, Fig. 2) produced an inhibitory effect on binding values. The same result was obtained with a number of normal sera. About 50% inhibition was observed when 20% of normal mink serum was included in the RIA incubation mixture. To minimize the inhibitory influence of normal serum, antibody detection assays were done in a buffer containing 1% normal mink serum. This eliminated the inhibitory effect when serum concentrations less than 1% were used. When serum concentrations higher than 1% were required to establish inhibition of binding (as the day 3 mink in Fig. 2), it was necessary to compare the observed inhibition with that of normal mink serum. There was an antibody titer increase of more than a factor of 10^5 when the day 3 mink serum was compared with the terminal diseased mink (>100).

Comparison of the sensitivities of different methods measuring antibody to ADV. The anti-ADV titers of 16 sera and one IgG preparation were measured by four different methods. These were RIA, direct countercurrent electrophoresis, indirect countercurrent electrophoresis, and fluorescence microscopy. The titers are presented in Table 1. Also indicated in Table 1 are total protein and γ-globulin content of the sera and the quantitation of specific antibody to ADV, based on the RIA titers.

The sensitivity of the ab-RIA was 10 ng of antibody per ml of incubation mixture. Since only 0.5 ml of incubation mixture was used in the RIA, the lowest amount of antibody detectable was 5 ng.

Different observations can be drawn from Table 1. Antibody titers from 5 to more than 10^6 were observed. There was a steady increase of antibody titer with time after infection. The serum pools from terminal diseased mink all had titers of more than 10^5, the serum pool from the non-Aleutian mink infected with the DK strain being the lowest. The ab-RIA was found much more sensitive than the other methods analyzed.

Quantitation of viral antigen detected by RIA. The previous experiments indicated that a sensitive RIA could be developed and related to a known amount of a standard antigen. We also attempted to estimate the amount of viral protein in such a preparation. Previous studies

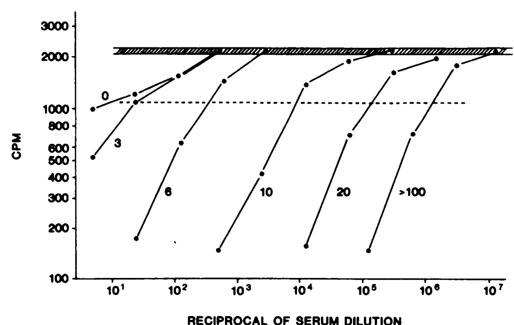

FIG. 2. Detection of antibody to ADV by inhibition of binding RIA. The hatched zone represents zero inhibition. The dotted line represents the 50% inhibition value. Each curve represents inhibition by one serum obtained the day indicated after experimental infection with Utah I ADV (0 means normal serum, >100 represents a pool of sera from plasmacytotic mink).

TABLE 1. Comparison of the sensitivities of different methods measuring antibody to ADV[a]

Serum no.	Virus strain	Day killed	Protein (mg/ml): Total serum protein	Protein (mg/ml): Serum γ-globulin	Protein (mg/ml): Anti-ADV[b] antibody	Titer: RIA[c]	Titer: Countercurrent electrophoresis[d] Direct	Titer: Countercurrent electrophoresis[d] Indirect	Titer: Fluorescence microscopy[d]
4674	Utah 1	3	70	5	0.00005	5	0	8	0
4675	Utah 1	6	69	6	0.004	360	4	32	0
4676	Utah 1	10	75	9	0.09	8,800	80	160	16
4650	Utah 1	10	71	6	0.12	12,500	40	1,600	4
4651	Utah 1	10	72	8	0.15	15,000	40	400	4
4647	Utah 1	12	67	6	0.03	3,200	80	800	16
4677	Utah 1	20	68	9	1.24	125,000	800	6,400	6,400
Pool	Utah 1	>100	90	34	13.43	1,350,000	800	16,000	800
4652	DK	10	65	7	0.02	2,300	8	160	<8
4653	DK	10	71	5	0.02	1,600	16	320	<4
Pool[e]	DK	>100	77	11	1.39	140,000	400	4,000	160
4648	Pullman	35	70	9	2.89	290,000	800	1,600	800
4649	Pullman	35	63	10	1.24	125,000	200	1,600	800
Pool	Pullman	>100	86	33	6.96	700,000	1,600	8,000	2,560
IgG[b]	Pullman	>100		11	1.69	170,000	160	3,200	80
Pool	ADV-G	>100	71	16	3.48	350,000	800	8,000	1,600
Pool[f]			63	6	0	0	0	0	0

[a] Titers are expressed as reciprocal of serum dilutions.
[b] The RIA findings from Fig. 1 indicated that 15.4% of the IgG preparation prepared from the anti-Pullman serum was specific for ADV. Based on this value, and assuming that the binding affinities between antibody and antigen are the same for all of antisera and IgG preparations investigated, the indicated concentrations of specific anti-ADV antibodies can be calculated. For example, a 1:170,000 dilution of 1.69 mg of specific antibody to ADV per ml inhibited 50% of the binding of 5 μl of Utah I antigen in the ab-RIA. A titer of 350,000, such as the anti-ADV-G serum pool exhibited, thus indicates a concentration of (350,000/170,000) × 1.69 mg/ml = 3.48 mg/ml of specific antibody to ADV in the anti-ADV-G serum pool.
[c] Measured as 50% inhibition of binding of 5 μl of ADV antigen. Fivefold dilution steps were performed.
[d] Twofold dilution steps were performed.
[e] Serum pool from non-Aleutian genotype mink.
[f] Normal serum pool.

indicated that standard ADV antigens contain both DNA and ferritin (1). Consequently, to reduce the level of these contaminants, 3.5 ml of Utah I standard antigens was DNase treated and was fractionated on a 40-ml Sepharose 6 B column in 0.05 M sodium carbonate (pH 9.6). Fractions (2 ml) were collected and analyzed for protein by measuring optical density at 280 nm and for ADV antigen by RIA. The fractionation curve is presented in Fig. 3. Fractions 6, 7, and 8 contained more than 80% of the ADV antigen activity, but only 0.1% of the total protein. The bulk of the ferritin eluted in fractions 10 to 17. Fraction 7 gave the highest specific activity in which 23.1% of the total antigen gave 0.065 units of optical density at 280 nm. Analyses of 50 μl of this fraction in sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed no bands visible after Coomassie blue staining. An optical density at 280 nm of 1.000 is regarded as an average estimate of a protein concentration of 1 mg/ml (i.e., when no other values are known). By using this value and by assuming that all of the protein represented in fraction 7 was virus protein, we estimated that the concentration of virus in the standard antigen preparation was 161 μg/ml. Obviously this estimate is conservative, and the real virus concentration could be much lower. Nevertheless, by using this estimate the ag-RIA detected as little as 3.2 ng of ADV antigen.

Included in the Fig. 3 insert are also protein analyses from a similar Utah I ADV fractionation experiment before and after DNase treatment of ADV. The absorbance peak was much higher with no DNase treatment and did not correlate with the ADV antigen peak found in the RIA, indicating that the virus in the void volume from the untreated fractionation was contaminated with nonviral components. On the other hand, DNase treatment of the ADV before Sepharose 6 B fractionation was shown to give counts per minute/optical density at 280 nm ratios (specific activities) which for fraction 7 and 8 were fairly close to each other (fraction 7 giving a little higher ratio than fraction 8), indicating a highly purified ADV antigen preparation.

ADV-antigen detection in different organs and cell types during infection. We next utilized the

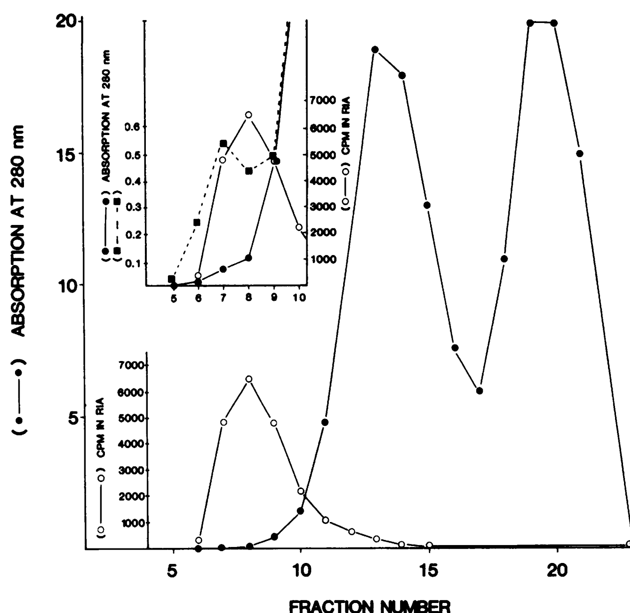

FIG. 3. Fractionation of 3.5 ml of Utah I standard antigen (DNase treated) by Sepharose 6B chromatography. A 40-ml column was used. Fractions of 2 ml were collected. The dotted curve in the insert indicates 280 nm absorption values from an identical fractionation experiment, with the exception that the Utah I antigen was not DNase treated.

RIA to detect viral antigen in infected mink. This analysis was complicated by the fact that some crude homogenates contained free antibody and masked the ADV antigen (data not shown). This observation was not surprising since viral antibody was detected as early as 3 days after experimental infection and since most of the organs containing viral antigens are either highly vascular or lymphoid in nature. Consecutive Freon treatments of the organs and cell types thus were performed until no antibody could be found. At this step the ag-RIA binding value was taken as the best ADV antigen estimate for that preparation. The results from this experiment are presented in Table 2. It should be noted that successive Freon treatment under these conditions did reduce the amount of ADV antigen (data not shown). Therefore the antigen concentrations given in Table 2 after several Freon treatments represent an underestimate of

TABLE 2. Detection of Utah 1 ADV antigen in organs and cell suspensions from infected mink[a]

Mink no.	Day after infection	RIA antibody titer[b]	ADV antigen (ng/mg of tissue)								
			Liver	Kidney	Spleen	Lymph node	Gut	Thymus	Lymphocytes	Peritoneal exudate cells	Bone marrow cells
4674	3	5	<3	4	<3	ND[c]	4	<3	<3	11	<6
4675	6	360	<3	16	12	ND	68	<3	<3	<11	64
4676	10	8,800	7	<3[d]	26	ND	<3[d]	<3	129	11	225
4647	12	3,200	62	64	507	233	ND	<3[d]	ND	150[d]	ND
4677	20	125,000	<3[d]	<3[d]	10[d]	ND	<3[d]	<3[d]	<3[d]	11[d]	<3[d]

[a] Tissues or cells were taken from sapphire mink. Organ homogenates representing from 4 to 20 mg of tissue and cell suspensions representing from 3×10^5 to 5×10^6 cells were tested in the RIA. The binding values obtained in the RIA were correlated to those of the standard antigen preparation (Fig. 1), and the amounts of ADV-antigen were calculated on the basis of 161 ng of ADV antigen per ml of standard antigen preparation (Fig. 3). For cell suspensions, 10^8 cells were assumed to equal 100 mg of tissue.

[b] Antibody titers are from Table 1.

[c] ND, Not determined.

[d] This preparation was exposed to several Freon treatments to become antibody negative. The indicated ADV concentration might represent an underestimate of the actual ADV antigen concentration present in vivo.

the actual ADV antigen concentration present in vivo.

Organs and cell preparations from normal mink similar to the preparations shown in Table 2 were also analyzed in ag-RIA and ab-RIA. Antigen concentrations higher than 3.2 ng/mg of tissue or antibody could not be detected in these preparations.

DISCUSSION

A one-step ag-RIA was developed for quantitation of ADV antigen. The two necessary reagents were solid-phase antiserum adsorbed onto plastic pearls and a [^{125}I]IgG preparation prepared from the same pool of antiserum. The two reagents are competitive for antigen, but when used in approximately the same concentrations a complex of solid-phase antibody–antigen-[^{125}I]IgG on the plastic pearls developed. The multivalency of the antigen (ADV has 32 or 64 capsomers) is possibly also critical for establishing such a complex.

The Utah I ADV was chosen as a standard virus antigen and a serum from mink infected with the Pullman isolate of ADV as the standard antibody source. This, we believe, is not critical for evaluating the results. No antigenic differences have clearly been demonstrated between the Utah I and Pullman strain of ADV; further, a recent publication by us (submitted for publication) shows that the anti-Pullman serum has more antibodies against the Utah I ADV than against the Pullman ADV.

We estimated that the ag-RIA could detect 3.2 ng of ADV antigen, but the actual sensitivity was probably higher, since the purity of the ADV preparation used for the sensitivity calculation (Fig. 3) could not be unequivocally assessed. It has, however, previously been shown by the use of crossed immunoelectrophoresis (1) that the major antigenic components present in similar antigen preparations were ferritin, ADV antigen, and DNA. In the present study, the DNA was digested and, along with ferritin, removed by Sepharose 6B chromatography (Fig. 3). Thus, we assumed ADV to be the major remaining component in the fractions used for the ADV antigen quantitation.

The Utah I standard antigen used in these studies represents a partially purified virus preparation; as a result, the RIA detected only antibodies against structural virion components. Since mink infected with ADV also have antibodies to a nonvirion protein (5), such antibodies would not be detected in RIA. An adaptation of the RIA utilizing antigen prepared from ADV-infected cell culture, however, would circumvent this problem.

Normal serum samples were found to cause some inhibition of binding when used in high concentrations in the RIA. There are several possible explanations for this. A change of the binding kinetics between antigen and antibody in very concentrated protein solutions is one possible explanation. Another explanation is that with the high sensitivity of the ab-RIA, a certain concentration of cross-reacting antibodies to ADV might be present in the normal serum, thus competing for antigen binding.

When the sensitivity of the ab-RIA was compared with that of other known methods for anti-ADV quantitation (Table 1), the RIA was shown to be the most sensitive with the indirect countercurrent electrophoresis as number 2, and with the fluorescence microscopy and the direct countercurrent electrophoresis as the less sensitive techniques. The latter two methods have previously been compared with regard to sensitivity, a study which showed the fluorescence microscopy to be a little more sensitive than the countercurrent electrophoresis (9). A good correlation was found between the different methods investigated. Being a fast, one-step, and sensitive method for detection of antibodies to ADV, we believe that this RIA could be a future alternative method for screening mink sera in ADV eradication programs. It should, however, be noticed that low-affinity antibodies against ADV would possibly not be measured by the present competitive ab-RIA.

Demonstration of ADV antigen in different organs and cell types during the early events of ADV infection was made possible by repeated Freon treatment of infected tissues (Table 2). The findings showed small amounts of virus antigen initially (day 3), primarily found in the gut, kidney, and peritoneal exudate cells. On day 6 the gut contained substantial antigen, as did bone marrow cells. After day 6 lymphoid organs (spleen and lymph nodes) were found to be the main antigen sources. Since many peritoneal exudate cells are macrophages, it was not surprising that such cells, with their highly developed phagocytic potential, contained antigen. The antigen found in liver could similarly be explained by the presence of phagocytic Kuppfer cells in this organ. ADV antigen in Kuppfer cells as measured by fluorescence microscopy has previously been described (14). The existence of ADV antigen in the kidney in relatively large amounts was demonstrated in one mink (4647) on day 12 after experimental infection. Although the main cause of mink death due to ADV infection is known to be glomerulonephritis (14), it was surprising to find so much antigen in the kidneys at this early stage of infection. It probably reflects immune complexes trapped in the glomeruli. Mink 4647 was the one in which the most ADV antigen could be

detected. This could be due to the relatively low titer of antibody found in this mink. There have been two major reports on ADV quantitation in mink organs. One study utilized infectivity titration in vivo of different organs from an ADV infected mink (10). Only the mesenteric lymph nodes, spleen, and kidneys could be demonstrated as sources of virus; the other organs could not be demonstrated to have larger quantities of ADV than blood. The other study used fluorescence microscopy for quantitation of ADV antigen (14). Most antigen was found in liver, spleen, and lymph nodes. Fluorescence was located in the red pulp of the spleen, medullary areas of the lymph nodes, and in hepatic Kuppfer cells, all suggesting macrophages to be the ADV-positive cells.

In the present study, only small amounts of antigen could be demonstrated on day 20. This could be explained by the fact that the peak of infectious Utah I ADV particles has been found on day 10 (14). From this day on there is a decline in infectious virus concentrations. On the other hand, since many Freon treatments were necessary to remove antibody from virus in the tissues from this mink, low amounts of antigen found might reflect excessive antigen loss during the treatment.

Since 15.4% of the IgG in the anti-Pullman ADV serum pool was ADV specific, the bulk of the increase of γ-globulin was not specific antiviral antibody. This implied that the hypergammaglobulinemia represented a polyclonal antibody response. Studies in progress utilizing nylon wool-fractionated lymph node cells suggest that ADV antigen is mainly found in the adherent cell population (predominantly B lymphocytes and macrophages; B. Aasted, M. Bloom, and R. Race, work in progress). These findings could be interpreted as an indication that B lymphocytes or pre-B lymphocytes might be infected by ADV and directly stimulated to produce a polyclonal response. Thus, ADV infection in mink might be similar to Epstein-Barr virus infection in humans, in which Epstein-Barr virus induces a polyclonal B cell activation in vitro and, in rare cases, in vivo (12, 16). This would indeed be very interesting, since nondefective parvoviruses, like ADV, are not known to transform infected cells.

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